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(54) Title: BACTERIAL CELL SURFACE PROTEIN WITH FIBRONECTIN, FIBRINOGEN, COLLAGEN AN LAMININ BINDING ABILITY, PROCESS FOR THE MANUFACTURE OF THE PROTEIN AND PR FYLACTIC TREATMENT

(57) Abstract

A cell surface protein having an ability of binding fibronectin, fibronogen, collagen, and or laminin, which prote is obtained by cultivating one or more bacterial strains having fibronectin, fibronogen, collagen, and/or laminin bindi properties on a suitable medium, isolation of such a strain, washing, decomposing of the strain, and purification of fibro ectin, fibronogen, collagen, and/or laminin binding component. The invention also refers to the production of the cell s face protein, the use thereof for prophylactic purposes, and prophylactic treatment of men and animals. A preferred e bodiment is hereby prophylactic treatment of ruminants against mastitis.

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Bacterial cell surface protein with fibronectin, fibrinogen, collagen and laminin binding ability, process for the manufacture of the protein and profylactic treatment.

DESCRIPTION

Technical field

The present invention relates to a cell surface protein having an ability of binding to fibronectin, fibrinogen, collagen, and/or laminin, process for uts preparation, as well as the use of such a cell surface protein.

The object of the present invention is to obtain a possibility of blocking fibronectin, fibrinogen, collagen, and/or laminin in a traumatic wound tissue in order to prevent adherence of pathogenic bacterial strains on fibronectin, fibrinogen, collagen, and/or laminin.

Background of the invention

Staphylococci and streptococci are usually often regarded as a group of gram positive bacteria, which develops purulent matter (pus) at infections, so called pathogenic cocci. This group does not only contain the classical Staphylococcus aureus and Streptococcus pyogenes (group A streptococcus), but also other staphylococci and streptococci, such as Staphylococcus epidermis, Staphylococcus haemolyticus, Staphylococcus hyicus, streptococci of Groups B, C, G, and H, viridans streptococci, etc. Even gram negative bacteria such as Escherichia coli can cause such infections.

These pathogenic bacterial strains causes different infections in man and in animals all the way from small selfhealing skin infections, to serious sepsis (blood infection). At the infection of animals by these strains the animals are not only suffering, but also great economical damages are caused to the owners of the animals due to production cut-off. Mastitis in milking cows is such an economically damaging infection.

In man such bacterial strains cause i.a. heart valve infections, but also other infections as the commonly known "hospital illness", i.e., most often an infection of an open wound, which shows difficulties in healing , can produce large amounts of pus, and can cause reoperation

Particularly, the heart valve infections threatens risk groups already exposed within the hospital care.

The term wound used means that normally covering epithel cellular layer, and other surface structures have been damaged by mechanical, chemical, or other influence. The term wound can hereby be divided into two main groups, viz: surface wounds, and deep wounds. The term surface wound means a trauma on the surface of the body or a surface in direct connection to the cavities of the body, i.e., the gastro-intestinal duct, mouth cavity, urethra, milk ducts, etc. The term deep wounds means trauma in the inner of a body caused by violent outer assault or by surgical incisions in different tissues.

When a wound is caused, fibronectin, fibrinogen, collagen, and/or laminin are exposed in the wound tissue. These proteins form together with so called proteoglucans a net work structure in different reinforcement tissues, and is the structure onto which connective tissue (fibroblasts) and epithel cells grow at a natural wound healing.

The natural wound healing can, however, be prevented by pathogenic bacteria colonizing therein, primarily by pyogenic cocci, and secondly by other pathogenic strains, such as E. coli and other gram negative rod shaped bacteria.

Examples of such a colonizing of a tissue damage are:
i) colonizing of wounds in skin and connective tissue, which wounds have been caused by a mechanical violence, chemical damage, and/or thermical damage;
ii) colonizing of wounds on mucuous membranes, such as in the mouth cavity, or in the mammalian glands, urethra, or vagina;
iii) colonizing on connective tissue proteins, which have been exposed by a minimal tissue damage (microlesion) in connection with epithel and endothel (mastitis, heart valve infection).

Description of the present invention.

It has now surprisingly been shown possible to isolate proteins from bacterial cell surfaces, which proteins adhere to fibronectin, fibrinogen, collagen and/or laminin, which cell surface proteins are derived

from bacterial strains mentioned above.

Such cell surface proteins can thereby be used for the treatment of wounds, e.g., for blocking protein receptors or for immunization (vaccination). In the latter case the body creates specific antibodies, which can protect against invasion of bacterial strains comprising such a cell surface protein. Hereby the antibodies block the adherence of the bacterial strains to a damaged tissue.

The characteristics of the present invention are evident from the accompanying claims.

By means of the present invention it is thus achieved that pathogenic bacterial strains can be effectively prevented from colonizing a traumatic wound tissue.

When using the present cell surface proteins for the purpose of immunization (vaccination) in mammals including man, the protein is dispersed in a sterile, isotonic saline solution, optionally while adding a pharmaceutically acceptable dispersing agent.

A suitable dosage to obatin immunization is 0.5 to 4 /ug of cell surface proteins per kg bodyweight and injection of immunization. In order to obtain a durable immunization, vaccination should be carried out at three consecutive occasions with an interval of 1 to 3 weeks. Furthermore, one carries out the immunization in accordance with science and tested practise.

When using the present cell surface proteins for topical, local application the protein is dispersed in an isotonic saline solution to a concentration of 25 to 200 /ug per ml. The wounds are then treated with such an amount only to obtain a complete wetting of the wound surface. For an average wound thus only a couple of millilitres of solution are used in this way. After treatment using the protein solution the wounds are suitably washed with isotonic saline solution or another suitable wound treatment solution.

Below, an immunization of young cows against mastitis is shown. Topi-

cal use of cell surface protein can also be used for preventing mastitis by treating udders/teats with a solution comprising cell surface proteins, which prevents pathogenic, mastitis-inducing organisms to adhere thereto.

In accordance with the invention a mixture of cell surface proteins with different binding properties can be used, particularly if the binding properties of an infecting, bacterial strain are unknown, and there is a great demand for a rapid prevention of a massive bacterial epidemic infection; or the infection is caused by a mixture of bacteria.

The invention will be described more in detail in the following with reference to some Examples.

Example

A strain of Staphylococcus aureus, which binds to fibronectin was grown on a liquid medium (TS-broth), trypticase-soya-extract (Oxoid, Ltd., England).

After finished growth the bacteria were isolated by centrifugation and were washed with a saline solution (0.9 % NaCl in water). The bacteria were then decomposed using a bacteriolytic enzyme (Lysostaphin^{R}, Sigma, 5 mg/litre of cell culture). Fibronectin binding components were isolated by affinity chromatography on immobilized fibronectin bound to a dextrane gel (Sepharose, CL-4B, cyanobromide activated). The fibronectin binding components were then eluated by adding chaotropic ions (e.g. NaSCN, KSCN) in an aqueous solution. The eluation can also be carried out using an acidic solution, acetic acid solution having pH<3.

Fibronectin binding components consisting of proteins having their molecular weights within the range of 11,000 to 165,000, preferably 40,000 to 165,000 were isolated. The proteins may comprise a carbohydrate residue, whereby, however, it is the protein residue which is fibronectin binding, which is shown by the fact that the effect is totally eliminated after a treatment using protease, or heating to 80 to 100^{o}C.

The amino acid composition of the protein components obtained is evi-

dent from the Table below:

TABLE

Amino acid	Residues per 1000 amino acids	
	M_w=165K	M_w=87K
Aspartic acid	146	134
Threonine	107	103
Serine	65	78
Glutamine	171	151
Proline	62	58
Glycine	79	84
Alanine	46	47
Cysteine[a)]	2.3	n.d.
Valine	78	86
Methionine[a)]	5.8	n.d.
Isoleucine	47	38
Leucine	40	46
Tyrosine	23	41
Phenylalanine	20	36
Tryptophane[b)]	24	31
Histidine	32	30
Lysine	63	66
Arginine	12	--

a) Amino acid determined after a performic acid oxidation of a sample

b) Amino acid calculated from an absorbance at 280 nm and tyrosine content.

n.d. = not determined

In the Example the affinity chromatography has been used for purification/isolation of the protein. Other biochemical separation methods are ion exchange chromatography, and molecular sieve; electrophoresis incl. isotacophoresis; electrofocusing.

A conventional cultivation of S. aureus gives a cell surface protein of the above. For an effecient industrial production of receptors for vac-

cine, and other care the gen needs to be cloned in a suitable organism in order to obtain high yields.

A purified fibronectin binding cell surface protein has proved to be immunogenous at the immunization of rabbit and ruminants, and has thereby developed formation of antibodies.

Test 1.

Vaccination of SRB-heifers (1:st calf cow) with a fibronectin binding protein in accordance with the Example above.

Three SRB-heifers (Swedish Red-and-White Cattle) were vaccinated subcutaneously in the thorax region using 400 µm of fibronectin binding component (M_w 165,000 and 87,000). These injections were repeated twice with 14 days inbetween. Antibody determinations in serum and in milk by means of ELISA-method (Enzyme Linked Immuno Sorbent Assay) showed a very potent immuno response determinable in large dilutions of milk and serum already at the moment for the second immunization.

Two weeks after the second injection, i.e., at the moment for the third immunization injection the immuno response was regarded as enough stimulated to carry out an experimental udder infection (mastitis) in the three animals. These three animals, as well as two control animals from the same stock were exposed to an experimental udder infection using a strongly udder pathogenic strain isolated from acute bovine mastitis (S. aureus) in order to develop mastitis in the five animals. The test was carried out by washing, dispersing in an isotonic saline solution and then injecting into the teat and udder cavity using a standardized injection technique, 500 bacteria from a bacterial cultivation grown in a broth medium (TS-broth).

The following results were obtained:

i) very sparse growth in certain milk samples from vaccinated cows, only;

ii) very high number of bacteria in most milk samples from non-vaccinated animals;

iii) cell count determinations showed generally low cell counts in the

vaccinated animals;
iv) cell count determinations showed generally high cell counts in the non-vaccinated animals;
v) the vaccinated animals produced unchanged volumes of milk;
vi) the non-vaccinated animals showed markedly decreased milking volumes (>10%);
vii) determination of acute phase reactants type "C reactive protein", and albumine in the vaccinated animals showed no change of the values obtained prior to the innoculation;
viii) determination of acute phase reactants type "C reactive protein", and albumine in the non-vaccinated animals showed strongly increased values.

The results obtained show that antibodies against fibronectin binding protein are secreted into udder and are present in local wound lesions in an amount enough to sterically preventing the surface receptors of an infecting bacterial strain to bind to exposed fibronectin in the udder tissue.

Test 2.
Blocking of an infection in an open skin wound by wound treatment using fibronectin binding cell surface protein from S. aureus.

Standardized wound damages (2x2 cm) were made on the back of pigs (20-25 kgs) using a so called dermatom. These wounds placed in two rows of 8 wounds on each side of the spine were subjected to a thermical damage (250°C, 3 min). After thermical treatment the wounds were covered with a sterile bandage for 1.5 hrs, whereupon the wounds were infected with S. aureus strain (SA 113(83A)). Prior to bacterial infection the wounds on one side of the spine were treated with fibronectin binding cell surface protein, according to the Example above, solved in a sterile isotonic saline solution (100 µg per ml of NaCl-solution). In wounds pretreated in this way the development of an infection was prevented by, at the same time, washing the wounds twice a day using a sterile isotonic saline solution. Non-treated wounds showed in the lesions, bad infections within 2 to 4 days although washing twice a day using NaCl-solution; infections which did not heal untreated with antibiotics during an observation period of one week.

The results of this experiment show that surface exposed fibronectin is blocked by pretreating lesions using 100 µg/ml in NaCl, in such a way that infections are prevented. Bacteria applied can easily be removed by rinsing which is impossible in wounds not treated with cell surface protein.

Besides fibronectin other connective tissue binding proteins have been detected in different microorganisms, which bind to those connective tissue structures present in man and animal, viz. collagen, and laminin according to the table below:

	Fibronectin	Collagen	Laminin
Staphylococci (different types)	+	+	-[1]
Streptococci (Group A, C, G, H, opt. B)	+	+	+
Escherichia coli	+	-[1]	+

1) not yet tested
+ denotes presence

Test 3.

The binding of Staphylococci to immobilized fibronectin - a model to simulate binding to traumatic tissue (surgical wounds and mastitis).

A polymer surface was treated with different serum proteins, such as albumine and fibronectin. The polymer surface was then incubated with the respective protein dispersed in a sodium phosphate buffered saline solution (0.2 M sodium phosphate, pH 7.4, and 0.145 M NaCl) for 2 hrs at ambient temperature. The polymer surface was then dried by blowing air using a fan. Then the treated surface was subjected to a Staphylococci (strain SA 113(83A)) in a buffer solution, and dispersed in the presence of bovine milk, respectively. Already after a couple of minutes an uptake of bacteria was determined in both these testing systems, while a surface treated in the same way using albumine in

the same, and in a 10-fold higher concentration of protein solution does not show an active bacterial uptake (untreated surface is however hydrophobic and binds staphylococci unspecific). The binding of strain SA 113(83A) can be inhibited by first incubating the bacteria with an antiserum obtained from rabbit vaccinated with a purified receptor protein.

Test 4.

In a similar way a surface has been treated with laminin, and then, as above, bacteria have been added, in this case a Group A streptococcus strain. Thereby it has been shown that the streptococcus strain binds to the surface.

Test 5.

A polymer surface was treated with fibronectin (immobilized) in accordance with Test 3 above. Then the surface was treated with a cell surface protein (M_w 87,000) of Example 1 above solved in a physiologic saline solution, 100 µg per ml. Then the surface was treated with a Staphylococci (strain SA 113(83A)) dispersed in a buffer solution (phosphate buffer, 0.2 M Na-phosphate, pH 7.4, and 0.145 NaCl). After the treatment with staphylococci the polymer surface was rinsed with a physiological saline solution for eliminating loosly attached bacteria. At a subsequent analysis it was determined that no active binding of the staphylococci had taken place. The analysis was carried out by determining bacterial cell mass ATP (adenosine triphosphate) by means of bioluminiscens technique. In short the analysis is carried out by incubating the polymer surface with 50 µl of 1.25 N trichloro acetic acid to extract cellular ATP. The amount of ATP is determined and compared with a standard curve for ATP in a Luminometer 1250 (LKB-Produkter, Bromma, Sweden).

Claims

1. Cell surface protein having an ability of binding fibronectin, fibrinogen, collagen, and laminin, characterized in that it consists of a protein obtained by cultivating a bacterial strain binding to fibronectin, fibrinogen, collagen, and/or laminin, which cultivation has been carried out on a solid or liquid medium, isolation of the bacterial strain thus cultivated, washing with a saline solution; decomposing the bacterial starain washed; purifying the component binding to fibronectin, fibrinogen, collagen and/or laminin.

2. Cell surface protein according to calim 1, characterized in that it consists of a protein thus obtained by cultivation of a fibronectin binding bacterial strain, whereby the protein comprises components with molecular weights in the range of 11,000 to 165,000, preferably 40,000 to 165,000.

3. Cell surface protein according to claim 1, characterized in that it consists of a protein thus obtained by cultivation of a collagen binding bacterial strain.

4. Cell surface protein according to claim 1, characterized in that it consists of a protein thus obtained by cultivation of a fibrinogen binding bacterial strain.

5. Cell surface protein according to claim 1, Characterized in that it consists of a protein thus obtained by cultivation of a laminin binding bacterial strain.

6. Process for the manufacture of cell surface protein having a fibronectin, fibrinogen, collagen, and/or laminin binding property, characterized in that a fibronectin, fibrinogen, collagen, and/or laminin binding bacterial strain is cultivated on a solid or liquid medium; that the bacterial strain thus cultivated is isolated and washed, whereupon it is decomposed; that fibronectin, fibrinogen, collagen, and/or laminin binding component then is purified by means of biochemical separation technique.

7. The use of a cell surface protein having fibronectin, fibrinogen,

collagen, and/or laminin binding properies at the manufacture of a prophylactic or therapeutically active, wound treatment agent being active against wound pathogenic bacterial strains having fibronectin, fibrinogen, collagen, and/or laminin binding properties.

8. Prophylactic treatment of wound lesions in man and animals using a prophylactical therapeutically active amount of a cell surface protein having fibronectin, fibrinogen, collagen, and/or laminin binding properties, to prevent the generation of infections caused by wound pathogenic bacterial strains.

9. Prophylactic treatment against infections caused by wound pathogenic bacterial strains having fibronectin, fibrinogen, collagen, and/or laminin binding properties, whereby a cell surface protein having fibronectin, fibrinogen, collagen, and/or laminin binding properties is injected at one or more occasions in an amount active enough to cause immunization by forming antibodies against such wound pathogenic bacterial strains.

10. Prophylactic treatment according to claims 8 or 9 for prophylactic treatment of ruminants against mastitis, characterized in that a fibronectin, fibrinogen, collagen, and/or laminin binding cell surface protein is used for topical and/or immunizing treatment.

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE85/00227

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC [4]

A 61 K 39/02, 35/74, C 07 K 15/04, C 12 P 21/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	A 61 K 39/02, /085, /09, 35/74; C 07 K 15/04; C 12 P 21/00
US Cl	424:92

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

SE, NO, DK, FI classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	J. Infect. Dis. Vol 143, 325-45 published 1981 (Beachey E. H.) "Bacterial Adherence: Adhesin-Receptor Interactions Mediating the Attachment of Bacteria to Mucosal Surfaces"	1-10
X,P	Chemical Abstracts, Vol 100 (1984), abstract No. 153 642, J. Biol Chem. 1984, 259(6), 3734-38.	1, 5, 6
X	Chemical Abstracts, Vol 97 (1982), abstract No. 106 722e, Infect. Immun. 1982, 37(2), 526-31	1, 2, 6
X	Chemical Abstracts, Vol 98 (1983), abstract No. 139 254c, J. Biol. Chem. 1983, 258(5), 3396-401	1, 2, 6
A	Chemical Abstracts, Vol 97 (1982), abstract No. 212 596b, J. Biol. Chem. 1982, 257(24), 14788-94	1, 2, 6

.../...

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
1985-08-27	1985 -08- 29
International Searching Authority	**Signature of Authorized Officer**
Swedish Patent office	Carl Olof Gustafsson

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [ ] Claim numbers 8-10, because they relate to subject matter not required to be searched by this Authority, namely:

Profycatic treatment of human or animal body

2. [ ] Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	MEDLINE, Dialog Accession Nr 1322045 (NLM Accesson Nr 84162045) J. Biol. Chem., March 25 1984, 259(6) p 3734-8	1, 5, 6
Y	MEDLINE, Dialog Accession Nr 0484228 (NLM Accession Nr 81214228), Immunobiology 1981, 158(4), p 330-7	
A	EP, A1, 89 938 (SVENSKA SOCKERFABRIKS AB) 28 September 1983, see example 8, and pages 4-5	1-7